AD NUMBER

AD811015

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; FEB 1967. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Releases Branch, Frederick, MD 21701.

AUTHORITY

Army Biological Defense Research Lab ltr dtd 28 Sep 1971

AD

811015

TECHNICAL MANUSCRIPT 366

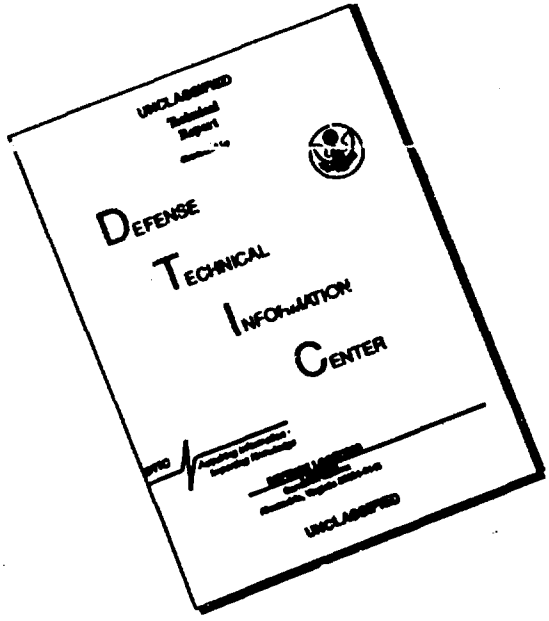
HISTOCHEMICAL β-GLUCURONIDASE DISTRIBUTION IN MAMMALIAN TISSUE AS DETECTED BY 5-BROMO-4-CHLOROINDOLE-3-YL-β-D-GLUCOPYRURONISIDE

Bjarne Pearson
Alfred C. Standen
John R. Esterly

FEBRUARY 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

DDC AVAILABILITY NOTICES

Qualified requesters may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 366

HISTOCHEMICAL β-GLUCURONIDASE DISTRIBUTION IN MAMMALIAN TISSUE AS DETECTED BY 5-BROMO-4-CHLOROINDOLE-3-YL-β-D-GLUCOPYRURONISIDE

Bjarne Pearson

Alfred C. Standen

John R. Esterly

Pathology Division
MEDICAL SCIENCES LABORATORY

Project 1L013001A91A

February 1967

ABSTRACT

A new substrate, 5-bromo-4-chloroindol-3-yl-β-D-glucopyruroniside, has been used to demonstrate β-D-glucuronidase in rat tissues. The reaction requires neither ferri-ferrocyanide nor a final coupling step. The optimal pH range is 4.0 to 5.4. The reaction is specific and can be readily distinguished from that of other glycosidases by inhibition with lactone. The wide tissue distribution observed is, in general, similar to that described in previous studies of tissue homogenates and histochemical studies. Because of the advantages of indoxyl substrates the method is applicable to the study of β-D-glucuronidase in pathological conditions.

I. INTRODUCTION

Indolyl substrates are useful for the detection of glycosidases in tissues. Tissue glycosidases hydrolyze the substrates by splitting the glycon and indoxyl moieties; the latter readily oxidizes to form an insoluble chromogenic final reaction product, the indigo; hence, no coupling reagent is required. The halogen-substituted compounds of this series give the most satisfactory final reaction product, particularly the 5-bromo-4-chloro- and the 5-bromo-6-chloro- compounds.[1,2] The former is preferable, but the 5-bromo-6-chloro- compound is also extremely useful because it results in a magenta color. Previously we have reported on the demonstration of β-D-glucosidase in tissue with this class of compounds and more recently on the β-D-galactosidase.[1,2]

This study reports the detection and localization of β-glucuronidase in tissue sections with a newly synthesized compound, the 5-bromo-4-chloroindol-3-yl-β-D-glucopyruronoside. The principle of the reaction is identical with that of the other glycoside substrates. β-D-glucuronidase in the tissue hydrolyzes this substrate to form the insoluble 5,5' bromo, 4,4' chlorobisindigo that is precipitated at enzymic sites.

II. MATERIALS AND METHODS

Tissues from the rat were studied with the halogen-substituted indoxyl substrate. Tissue blocks of approximately 2 cm^3 were quickly frozen in an acetone and dry ice bath (-70 C). The specimens were stored at -70 C until used. Fresh cryostat sections were cut and fixed for 20 minutes at 4 C in a solution of buffered 2.5% glutaraldehyde. The buffer was prepared by dissolving 0.2 g KCl, 0.2 g KH_2PO_4, and 1.15 g Na_2HPO_4 in 1000cc of 0.085 N NaCl. The slides were rinsed three times for 5 minutes each at 4 C in a solution of gum sucrose (17% gum arabic in 0.88 M sucrose). The slides were then incubated for 6 hours at 37 C in a solution containing 16 mg of 5-bromo-4-chloroindol-3-yl-β-D-glucopyruronoside,* 0.5 cc of 0.015 M NaCl, 0.5 cc of 0.015 M $MgCl_2$, 8 mg of spermidine trihydrochloride, and 30 cc of 0.1 M acetate buffer at pH 4.8. The slides were mounted in glycerogel or passed through alcohols and xylene and mounted in balsam with or without counterstains.

* Synthesized by Dr. Herman Plaut and staff at Cyclo Chemical Incorporated, 1922 E. 64th Street, Los Angeles, California 90001.

III. RESULTS

A. HYDROLYSIS

The histochemical hydrolysis of the substrate was readily identified in tissue sections by the fine bluish-green precipitate of the final reaction product (Fig. 1). Successful reactions occurred in sections of both liver and preputial glands over the pH range of 4.0 to 5.4, but values near pH 4.8 yielded the best results. Incubation at room temperature, even over longer periods, was less satisfactory than at 37 C.

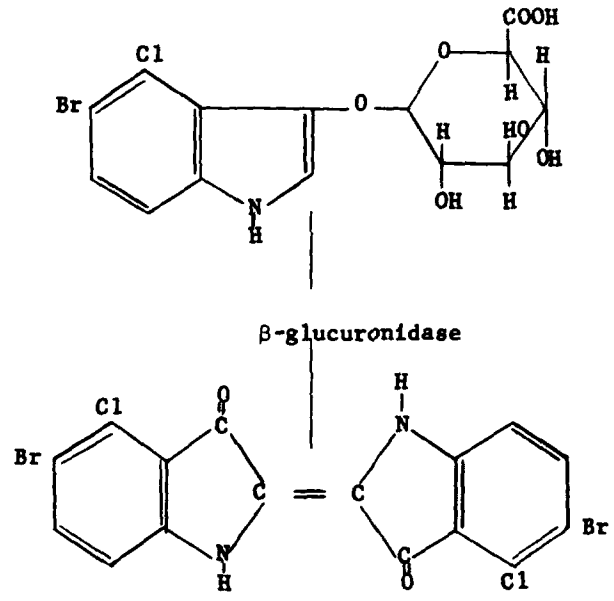


Figure 1. The Substrate Compound is an Indolyl Glucuronide, 5-Bromo-4-Chloroindol-3-yl-β-D-Glucopyruronside, that is Soluble and Nonchromogenic. The β-glucuronidase in tissue hydrolyzes this compound to form a fine bluish precipitate, 5,5' bromo-4,4' chloroindigo, which precipitates at enzymic sites in cells and tissue.

B. DISTRIBUTION OF β-D-GLUCURONIDASE

Tissues examined with this substrate reflected marked enzyme activity in the female preputial gland, liver, spleen, lymph node, adrenal, bladder, stomach, and small and large intestine. Less intense activity was noted in the lung, thyroid, salivary gland, kidney, and vagina. Minimal to trace reactions were detected in the uterus, ovary, epididymis, prostate, testes, thymus, and in decreasing order in the cerebrum, cerebellum, and posterior pituitary.

The preputial gland contained a high enzyme concentration. Indigo deposition could be seen after 5 to 15 minutes of incubation, and optimal reactions were obtained within 1 to 2 hours. The intensity, which varied in different portions of the gland, was related to the developmental stages of its ductal system. The cytoplasmic reaction was most intense in the larger and older ducts where cellular breakdown and accumulated secretion were prominent. The reaction was less intense in the younger and less mature portions of the ductal system, where it could be seen as discrete cytoplasmic rods and granules. The basement membrane appeared to stain, but the stroma was entirely negative except for strong reactions in occasional reticular cells (Fig. 2).

The liver also gave a strong reaction. The hepatic cells showed fine, particulate, rounded and elongated structures in the cytoplasm; Kupffer cells were positive, but the reaction was less prominent than in parenchymal cells (Fig. 3 and 4). The central veins, bile ducts, and arteries were negative. Under conditions of low substrate concentration or limited incubation time, a variable pattern of periportal staining was noted, but this was not present with optimal conditions.

The spleen demonstrated strong reactions in reticular cells surrounding lymphoid follicles and, to a lesser degree, in the marginal zone. The central zone was negative. Reticular cell staining was intense. In the marginal zone the cytoplasm showed discrete round and rod-like granules clustered around the nuclear membrane. The mesenteric lymph node showed a marked reaction in monocytes lying free in the sinusoids. Small round granules were diffusely scattered through the cytoplasm. The lymphoid cells of the medullary cords showed less reactivity, and where lymphoid follicles were developed, the central zone was negative.

The adrenal gave a strong positive reaction in the cortex. The medulla was negative. The glomerular zone gave the greatest reaction, followed by that in the reticular zone.

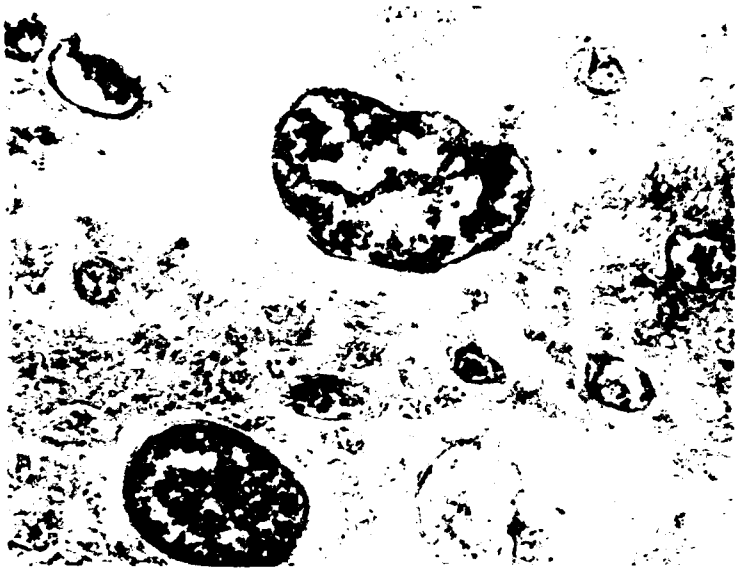


Figure 2. Fresh-Frozen Cryostat Section of Preputial Gland Incubated for 15 Minutes in a Solution Containing 5-Bromo-4-Chloroindol-3-YL-β-D-Glucopyruroniside Shows the Intensity of Reaction in Cells Lining and Accumulating in the Larger Ducts. With increase in incubation times, the medium sized and smaller ducts give the same reaction. No ferri-ferrocyanide was used in substrate. No counterstain was used. Substrate solution was used at pH 4.8. 250X.

Figure 3. Low-Power Magnification of Liver Showing Uniform Distribution of β-Glucuronidase in Hepatic Cells. No differences can be seen in enzyme activity in periportal area to the right and central vein area to left, or in midzonal area. No ferri-ferrocyanide was added. The pH was 4.8. Section was lightly counterstained with hematoxylin and picric acid. Optimal incubation time 4 hours. 100X.

Figure 4. High-Power Magnification of Liver in Figure 3 Showing β-Glucuronidase in Cytoplasm. Fine rod-like and granular precipitate of final reaction products can be seen. 750X.

The reticular cells of the lamina propria throughout the gastrointestinal tract gave a strong positive reaction. The stomach gave the strongest reaction; positive cells were particularly prominent in the region of the junction of the glandular and squamous portion of its epithelium (Fig. 5). The duodenum was similar to the jejunum; in the ileum, cecum, and descending colon the number of positive-staining cells diminished in the order listed. In addition, a diffuse but less marked staining was noted in the epithelial cytoplasm; goblet cells were negative. The reaction was present in the cytoplasm of the cells in the form of small round granules. This occurred in cells similar to those described in the stomach.

The bladder showed a strong reaction in the form of small round granules in the epithelial cytoplasm. The strongest staining was noted in the basilar layers of the epithelium. At the junction of the epithelium and the lamina propria there was intense activity. The positive areas were elongated irregular forms, some of which could be traced to histiocytes, but others could not be identified with certainty.

The following organs showed moderate enzyme activity. Reactivity in the lung was limited to the mucosa of the bronchi and some alveolar macrophages. Only the follicular epithelium of the thyroid was positive. The salivary gland revealed moderate activity in the epithelium of the ductal system, but the acini and other structures were negative (Fig. 6). Activity in the esophagus occurred in the basal cells of the epithelium. In the skin, staining was most prominent in the stratum granulosum. The vagina showed a reaction in the epithelium, but the strongest area was in the superficial layer. Only the cortex of the kidney was positive, the medulla and glomeruli were negative. The proximal tubules gave the best reactions, although irregularities occurred that were not readily explicable.

A number of organs showed only minimal or trace reactions. The ovary showed a positive reaction in the granulosa cells as well as the theca interna and externa. In the uterus a positive reaction was present in the endometrial lining and glands and in certain cells scattered in the stroma. The ductal epithelium of the epididymis gave a positive reaction. The testes, prostate, cerebrum, cerebellum, and posterior portion of the hypophysis were negative or showed only faint traces of enzymic activity. Under conditions employed for this substrate and incubation times, no reactivity was found in muscle, nerves, or vessels.

C. REACTION SPECIFICITY

No reaction product was visualized when the tissue section was subjected to boiling water or incubated in the presence of versene. β-D-glucuronidase activity was inhibited by 1,4-glucuronolactone but not by the lactones of galactose or fucose (Table 1).

Figure 5. Fresh-Frozen Cryostat Section of Junction of Squamous and Glandular Epithelium of the Stomach of Rat. The reaction for β-glucuronidase is strong and can be seen in the reticular cells of the lamina propria of the glandular portion and submucosa of the squamous portion. No ferri-ferrocyanide was used. Incubation time 5½ hours. 75X.

Figure 6. Fresh-Frozen Cryostat Section of Salivary Gland. There is intense reaction of β-glucuronidase that is most intense in cells lining the ducts. No ferri-ferrocyanide was used. Incubation time was 5½ hours. No counterstain. 105X.

TABLE 1. ANALOGUE INHIBITION OF GLYCOSIDASES

Substrate Compound	Enzyme Detected	Enzyme Inhibitor[a/]			
		None	1-4-Glucuronolactone	1-4-Galactonolactone	Fuconolactone
I	β-D-glucuronidase	+	-	+	+
II	β-D-fucosidase	+	+	+	-
III	β-D-galactosidase	+	+	-	+

a. + Visual intensity.
- Negative reaction.

IV. DISCUSSION

Difficulties with substrate synthesis, instability, and diffusion have been encountered in the development of methods for the detection and localization of glucuronidase activity in tissue sections. Previously described histochemical techniques for β-D-glucuronidase are the naphthol method developed by Seligman[3] and the Fishman-Baker procedure.[4] Both require coupling agents to yield a colored reaction product. The final step of the latter procedure is essentially a Prussian blue reaction, the validity of which has been challenged by Janigan and Pearse.[5] More recently another method, published by Pugh and Walker, used anilides of 2-naphthol-3-carboxylic acid with diazonium salts.[6] A coupling reagent is also required. The present method, using 5-bromo-4-chloroindol-3-yl-β-D-glucopyruroniside, needs no final coupling step because the hydrolyzed indoxyl(5-bromo-4-chloroindoxyl) readily forms the corresponding indigo, which is relatively insoluble and strongly adherent to protein.

Varying degrees of β-D-glucuronidase activity are present in the majority of the body tissues. β-D-glucuronidase demonstrated with the indoxyl substrate is similar to the findings of Seligman and Fishman.[3,4] The most conspicuous differences are those of the cerebrum and the cerebellum. We have found negative reactions or at the best only faint traces; the strong reactions described by Fishman[4] could not be demonstrated. In the epididymis, where a strong reaction was previously observed, we found only a minimal reaction. Similarly, we found no reaction in skeletal muscle. Sections of endocrine organs and tissues susceptible to hormonal changes showed a wide range of activity. The adrenal gave strong reactions but only minimal or trace reactions were found in the pancreatic islets. Clearly, results such as these in presumably normal animals will vary with the endocrine status of the host.

Tissue homogenates have been assayed for β-D-glucuronidase activity by several methods on many occasions. The results of these numerous studies have been collected and critically reviewed by Levvy and Marsh.[7] Our histochemical observations are in remarkable agreement with these biochemical data.

In common with other glycosidases, the reaction described is highly specific. Data from competitive inhibition studies with lactones clearly demonstrate the importance of the 6-carbon carboxyl group. Glucuronolactone with a carboxyl group in this position completely inhibited enzyme activity, but similar lactones, lacking a carboxyl on the sixth glycoside carbon (galactonolactone and fuconolactone) did not inhibit the reaction.

LITERATURE CITED

1. Pearson, B.; Andrews, M.; Grose, F. 1961. Histochemical demonstration of mammalian glucosidase by means of 3-(5-bromoindolyl)-β-D-glucopyranoside. Proc. Soc. Exp. Biol. Med. 103:619-623.

2. Pearson, B.; Wolf, P.; Vazquez, J. 1963. A comparative study of a series of new indolyl compounds to localize β-galactosidase in tissue. Lab. Invest. 12:1249-1259.

3. Seligman, A.M.; Tsou, K.C.; Rutenberg, S.H.; Cohen, R.B. 1954. Histochemical demonstration of β-D-glucuronidase with a synthetic substrate. J. Histochem. Cytochem. 2:209-229.

4. Fishman, W.; Baker, J. 1956. Cellular localization of β-glucuronidase in rat tissues. J. Histochem. Cytochem. 4:570-587.

5. Janigan, D.T.; Pearse, A.G.E. 1962. The mechanism of tissue staining by ferric hydroxyquinoline methods for β-glucuronidase. J. Histochem. Cytochem. 10:719-730.

6. Pugh, D.; Walker, P. 1961. Histochemical localization of β-glucuronidase and N-acetyl-β-glucosamidase. J. Histochem. Cytochem. 9:106.

7. Levvy, G.A.; Marsh, C.A. 1959. Preparation and properties of β-glucuronidase. Advances Carbohyd. Metab. 14:381.

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1 ORIGINATING ACTIVITY (Corporate author)	2a REPORT SECURITY CLASSIFICATION
Department of the Army Fort Detrick, Frederick, Maryland 21701	Unclassified
	2b GROUP

3 REPORT TITLE

HISTOCHEMICAL β-GLUCURONIDASE DISTRIBUTION IN MAMMALIAN TISSUE AS DETECTED BY 5-BROMO-4-CHLOROINDOLE-3-YL-β-D-GLUCOPYRURONISIDE

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5 AUTHOR(S) (Last name, first name, initial)

Pearson, Bjarne (NMI)
Standen, Alfred C.
Esterly, John R.

6 REPORT DATE	7a TOTAL NO. OF PAGES	7b NO. OF REFS
February 1967	14	7

8a CONTRACT OR GRANT NO.	9a ORIGINATOR'S REPORT NUMBER(S)
b PROJECT NO. 1L013001A91A	Technical Manuscript 366
c	9b OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d	

10 AVAILABILITY/LIMITATION NOTICES

Qualified requesters may obtain copies of this publication from DDC.
Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11 SUPPLEMENTARY NOTES	12 SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland 21701

13 ABSTRACT

A new substrate, 5-bromo-4-chloroindol-3-yl-β-D-glucopyruroniside, has been used to demonstrate β-D-glucuronidase in rat tissues. The reaction requires neither ferri-ferrocyanide nor a final coupling step. The optimal pH range is 4.0 to 5.4. The reaction is specific and can be readily distinguished from that of other glycosidases by inhibition with lactone. The wide tissue distribution observed is, in general, similar to that described in previous studies of tissue homogenates and histochemical studies. Because of the advantages of indoxyl substrates the method is applicable to the study of β-D-glucuronidase in pathological conditions.

14. Key Words

*Histochemistry
*Glucuronidase
Tissues
Glucosides
Rats
Detection

DD FORM 1 JAN 64 1473

Unclassified

Security Classification